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(57) Abstract

RANTES mutants characterised by the substitution or addition of amino acids at the N–terminal of RANTES wild–type sequence and in the N–loop and/or 40's loop regions of RANTES wild–type sequence, and their use as anti–HIV, anti–allergic or anti–inflammatory agents.

RANTES MUTANTS AND THERAPEUTIC APPLICATIONS THEREOF

The present invention provides RANTES mutants with reduced pro-inflammatory activity, increased HIV-suppressive activity, and antagonistic activity to wild-type chemokines.

Chemokines are small proteins involved in inflammatory mechanisms and in physiologic circulation of hemopoietic cells. Several studies have shown the important role of chemokines in recruiting leucocytes in inflammatory and autoimmune diseases, like rheumatoid arthritis, or during allergic reactions, like in asthma (Schall, T.J. The chemokines. In: The cytokine handbook, A Thompson ed. Academic Press, New York, 1994, p.419-460). Furthermore, some chemokines have been recently identified as potent natural inhibitors of human immunodeficiency virus (HIV) infection (Science 270, 1811-1815, 1995). Chemokines activity is due to their interaction with receptors having different specificity and expressed on the cell surface. Some of these receptors function as co-receptors for HIV-virus (Science 272, 872-877, 1996; Science 272, 1955-1958, 1996). The differential use of such co-receptors, particularly CCR5 the specific receptor for RANTES, MIP-1α and MIP-1β, and CXCR4, the SDF-1 specific receptor, represents a major determinant of the biological diversity among HIV strains. HIV-1 strains unable to infect continuous CD4+ T-cell lines, commonly involved in viral transmission and predominating during the asymptomatic phase of the infection, use primarily CCR5 as a co-receptor and are invariably sensitive to inhibition by CCR5-binding chemokines (Nature Med., 3:1259-1265, 1997). The most effective such chemokine, RANTES, is

therefore under investigation for the development of novel anti-HIV therapies (Nature, 383: 400, 1996). RANTES is a chemokine which belongs to the C-C family and is 68 amino acids long. Its sequence has been reported in J. Immunol. (1988).

WO 96/17935 discloses RANTES molecules which are modified at the N-terminus through the addition of an amino acid such as methionine, leucine or glutamine, as antagonists of RANTES or MIP-1α. In particular, the use thereof for the treatment of asthma, allergic rhinitis, atopic dermatitis, atheroma-atherosclerosis or rheumatoid arthritis is described.

Further, Elsner J. et al.. in "European Journal of Immunology, Vol. 27, 2892-2898 (1997)", and WO 96/17934, disclose the antagonistic activity of the Met-RANTES peptide.

The use of wild-type RANTES and of other chemokines of the same family in the treatment of allergic diseases, has been also described in WO 94/07521 and WO 94/21277.

WO 97/25350 discloses disaggregated mutants of MIP-1α or LD78 having HIV suppressive activity, whereas WO 98/13495 discloses human RANTES mutants unable to aggregate under physiologic ionic strength and which exhibit antiviral activity. Surprisingly now, it has been found that the addition of at least one amino acid at the N-terminus, and/or the substitution of one or more amino acids in the N-terminal region comprised between amino acids 1 and 11 of the mature form of the human chemokine RANTES, and/or in the "40's-loop" region, extending from Thr 43 to Asn 46, provides a notably higher efficacy towards different HIV isolates, both in primary mononucleated blood cells and in macrophages, a reduced pro-inflammatory activity and a potent antagonistic activity, as compared to the wild-type molecule. In

particular, the mutants of the invention competitively antagonise wild-type RANTES, MIP-1α or MIP-1β, and, with a comparable mechanism, the interaction between the HIV virus and a chemokine receptor. Preferably, one or more of the amino acids: Ser 1, Ser 4, Ser 5, Tyr 3, Asp 6, Tyr 14, Arg 17, Arg 44, Lys 33, Lys 45 and Arg 46 are mutated, with respect to the wild-type human form described in J. Immunol. 141:1018-1025, 1988, as reference molecule. Preferably, the amino acids Ser 1, Ser 4, Ser 5, Tyr 3 are replaced by neutral or hydrophobic amino acids, Asp 6 is replaced by a positively charged amino acid, Tyr 14 by a hydrophobic aromatic, Arg 17, Lys 33, Arg 44, Lys 45 and Arg 46 by a small sized hydrophobic amino acid.

The following mutations are more preferred: Ser 1 with Cys, Ser 4 with Cys, Ser 5 with Cys, Tyr 3 with Ala, Asp 6 with Arg, Tyr 14 with Phe, Arg 17, Lys 33, Arg 44, Lys 45 and Arg 46 with Ala. A first group of mutants according to the invention is characterised by a triple mutation selected from a) Ser 1 with Cys; Ser 5 with Cys; Asp 6 with Arg, or b) Ser 1 with Cys; Ser 5 with Cys; Arg 17 with Ala, or c) Ser 1 with Cys; Ser 5 with Cys; Arg 44 or Lys 45 or Arg 46, with Ala. A second group is characterised by a double mutation selected from a) Ser 1 and Ser 5 with Cys, or b) Ser 1 and Ser 4 with Cys, or c) Ser 1 with Cys and Arg 44 with Ala, or d) Asp 6 with Arg and Arg 44 with Ala. A third group is characterised by a single mutation selected from a) Ser 1 with Cys, b) Tyr 3 with Ala, c) Asp 6 with Arg, d) Tyr 14 with Phe, e) Arg 17 with Ala, f) Lys 33 with Ala, g) Arg 44 with Ala, h) Lys 45 with Ala, i) Arg 46 with Ala. Furthermore, the above mutants can be added with up to two amino acids at the N-terminal, which are preferably selected from Leu, Ala, Cys or Trp. For example, Ser 4 may be replaced by Cys and

simultaneously an additional Cys may be added at the N-terminus. In particular, the single mutant Cys 1 or –1, which contains a free –SH group, may represent an optimal substrate for further chemical modifications.

According to other aspects, the invention provides wild-type RANTES, having no internal amino acid mutations but bearing an additional amino acid at the N-terminus, which is preferably Cys, said RANTES derivatives being endowed with anti-HIV and anti-inflammatory activity, and the use of wild-type RANTES added with a Leu at the N-terminus (Leu(0) RANTES) as anti-HIV agent.

It is possible that the properties of some mutants according to the invention, in particular those carrying 1 or 2 additional Cys, are determined by structural modifications due to the formation of a new disulphide bond. Considering the structure of RANTES (Biochem. 1995, 34:9307-9314) or the structure of homologous molecules like SDF-1 (EMBO J., 16:6996:7007, 1997), it is also possible that the N-terminal or N-loop regions contribute to form the three-dimensional site of interaction with the specific membrane receptor.

According to another aspect, the invention provides for peptides corresponding to RANTES fragments in the N-terminal, N-loop and/or "40's-loop" regions, said peptides contain the described mutations and competitively antagonise wild-type RANTES, MIP-1α or MIP-1β, or the interaction between HIV virus and a chemokine receptor.

According to other aspects, the invention provides nucleotide sequences encoding for the described mutants, the expression vectors comprising such nucleotide sequences, chimeric or fusion proteins which comprise a sequence corresponding to the invention mutants and a carrier

sequence, for example a sequence aimed at improving the pharmacokinetic properties of active peptides or proteins; furthermore, the invention provides the use of such RANTES mutants as anti-HIV agents as well as anti-inflammatory, anti-allergic or anti-asthmatic agents.

By the term RANTES, any polypeptide functionally equivalent to the human RANTES is meant, as well as equivalent proteins derived from cross-reactive species, as well as variants and allelic forms thereof which may differ from the standard sequence reported in J. Immunol. 141:1018-1025, 1988.

The mutants of the invention may be prepared by conventional techniques of DNA cloning, recombination and in vitro expression, using suitable synthetic oligonucleotides, for example with techniques of site-directed mutagenesis or by the DNA Polymerase Chain Reaction (PCR). The resulting DNA is then inserted into an appropriate expression vector for a prokaryotic or an eukaryotic host. Alternatively, mutants can be prepared according to conventional methods of peptide synthesis.

For the envisaged therapeutical purposes, the mutants of the invention will be administered in form of suitable pharmaceutical compositions by the parenteral, sublingual, intranasal, inhalatory or topical route of administration, prepared according to conventional techniques, which are suitable for polypeptide or protein active substances.

The amount of polypeptide to administer will be sufficient to cause a significant inhibition of HIV infection or replication, or reduction of inflammatory responses, such as in rheumatoid arthritis, or in degenerative diseases such as atherosclerosis, or in allergic diseases such as asthma, rhinitis and dermatitis. The specific dosage will be determined

CLAIMS

1. A RANTES mutant wherein, as compared to human wild-type RANTES, at least one amino acid is mutated in the N-terminal region, in the N-loop region, in the 40's-loop region or in all the three regions, said mutant having the capability to competitively antagonise wild type RANTES, MIP-1α or MIP-1β, or to antagonise the interaction between HIV virus and a chemokine receptor.

2. A RANTES mutant according to claim 1, wherein one or more of the following amino acids is/are mutated: Ser 1, Tyr 3, Ser 4, Ser 5, Asp 6, Tyr 14 Arg 17, Arg 44, Lys 45,.

3. A RANTES mutant according to claim 2, wherein the mutation is selected from:

a) Ser 1 with a neutral or hydrophobic amino acid;

b) Ser 4 with a neutral or hydrophobic amino acid;

c) Ser 5 with a neutral or hydrophobic amino acid;

d) Tyr 3 with a neutral or hydrophobic amino acid;

e) Asp 6 with a positively charged amino acid;

f) Tyr 14 with a idrophobic aromatic amino acid;

g) Arg 17 with a small-sized hydrophobic amino acid;

h) Arg 44 with a negatively charged or a small hydrophobic amino acid;

i) Lys 45 with a negatively charged or a small hydrophobic amino acid;.

4. A RANTES mutant according to claim 3, wherein said mutation is selected from:

a) Ser 1 with Cys;

b) Ser 4 with Cys;

c) Ser 5 with Cys;

d) Tyr 3 with Ala;

e) Tyr 14 with Phe;

f) Asp 6 with Arg;

g) Arg 17 with Ala;

h) Arg 44 with Glu or Ala;

i) Arg 45 with Glu or Ala;

5. A RANTES mutant according to claim 4, comprising a triple mutation selected from :

a) Ser 1 with Cys; Ser 5 with Cys; Asp 6 with Arg;

b) Ser 1 with Cys; Ser 5 with Cys; Arg 17 with Ala;

c) Ser 1 with Cys; Ser 5 with Cys; Arg 44 with Glu or Ala;

6. A RANTES mutant according to claim 4, comprising a double mutation selected from:

a) Ser 1 with Cys; Ser 5 with Cys;

b) Ser 1 with Cys; Ser 4 with Cys;

c) Ser 1 with Cys; Arg 44 with Glu or Ala;

d) Asp 6 with Arg; Arg 44 with Glu or Ala;

7. A RANTES mutant according to claim 4, comprising a single mutation selected from:

a) Tyr 3 with Ala;

b) Ser 1 with Cys;

c) Asp 6 with Arg;

d) Tyr 14 with Phe

e) Arg 17 with Ala;

f) Arg 44 with Glu or Ala;

g) Lys 45 with Glu or Ala.

8. A RANTES mutant according to anyone of the preceding claims, further comprising one or two additional amino acids at the N-terminal.

9. A RANTES mutant according to claim 8, wherein said additional amino acids are selected from Leu, Ala, Cys, and Trp.

10. A RANTES mutant according to claim 9, wherein said additional amino acid is Leu.

11. A RANTES mutant according to claims 8-9, wherein Cys is added at the N-terminal and Ser 4 is mutated into Cys.

12. A RANTES mutant according to claims 8-9, wherein Cys is added at the N-terminal and Ser 5 is mutated into Cys.

13. A peptide derived from RANTES sequence of N-terminal, N-loop or 40's loop regions comprising the mutation or addition of claims 1-11, having the ability to competitively antagonise wild type RANTES, MIP-1α or MIP-1β, or to antagonise the interaction between HIV virus and a chemokine receptor.

14. Wild-type RANTES added with an amino acid at the N-terminus, wherein said amino acid is Cys.

15. A nucleotide sequence encoding a RANTES mutant of claims 1-12.

16. A vector for eukaryotic or prokaryotic expression comprising the sequence of claim 15.

17. A pharmaceutical composition having HIV-inhibiting, antiallergic, antiasthmatic or anti-inflammatory activity, comprising a mutant of claims 1-12 or the RANTES derivative of claim 14 as the active ingredient.

18. A pharmaceutical composition having HIV-inhibiting, antiallergic,

antiasthmatic or anti-inflammatory activity, comprising a peptide of claim 13 as the active ingredient.

19. A process for preparing RANTES mutants of claims 1-12 which comprises culturing eukaryotic cells trasfected with vectors containing DNA fragments encoding said mutants.

20. A process according to claim 19, wherein said vector is a baculovirus expression vector.

21. A process according to claim 19, wherein said vector is a E. coli expression vector.

22. The use of wild-type RANTES added with a residue of Leu at the N-terminus (Leu(0) RANTES), for the preparation of a medicament having anti-HIV activity.